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(54) **Title:** USE OF NSAIDs FOR THE TREATMENT OF PANCREATIC CANCER

(57) **Abstract:** The invention provides a method comprising the use of non-steroidal antiinflammatory drugs (NSAIDs), particularly sulindac or its analogs to treat pancreatic cancer.

USE OF NSAIDs FOR THE TREATMENT OF PANCREATIC CANCER

Background of the Invention

Cancer of the pancreas ranks just behind lung cancer, colon cancer, and breast cancer as the most common cause of death by cancer (1). It is more common among men, and men between the ages of 60 and 70 are most at risk. The cause of pancreatic cancer is unknown.

The most common symptoms are weight loss, abdominal pain, and jaundice. Weight loss, the causes of which are not fully understood, usually is significant. The average loss is about 25 pounds. Jaundice occurs if the cancer blocks the common bile duct. The survival rate with pancreatic cancer is poor. By the time the malignant tumor is identified, it often has spread (metastasized) to other parts of the body. The median survival is little more than six months from the time of diagnosis.

Often the tumor cannot be removed by surgery, either because it has invaded vital structures that cannot be removed or because it has spread to distant sites. Chemotherapy and radiation therapy can be used on the tumor, although these treatments often are not beneficial.

The number of nonsteroidal anti-inflammatory drugs (NSAIDs) has increased to the point where they warrant separate classification. In addition to aspirin, the NSAIDs available in the U.S. include meclofenamate sodium, oxyphenbutazone, phenylbutazone, indomethacin, piroxicam, sulindac and tolmetin for the treatment of arthritis; mefenamic acid and zomepirac for analgesia; and ibuprofen, fenoprofen and naproxen for both analgesia and arthritis. Ibuprofen, mefenamic acid and naproxen are used also for the management of dysmenorrhea.

The clinical usefulness of NSAIDs is restricted by a number of adverse effects. Phenylbutazone has been implicated in hepatic necrosis and granulomatous hepatitis; and sulindac, indomethacin, ibuprofen and naproxen with hepatitis and cholestatic hepatitis. Transient increases in serum aminotransferases, especially alanine aminotransferase, have been reported. All of these drugs, including aspirin, inhibit cyclooxygenase, that in turn inhibits

synthesis of prostaglandins, which help regulate glomerular filtration and renal sodium and water excretion. Thus, the NSAIDs can cause fluid retention and decrease sodium excretion, followed by hyperkalemia, oliguria and anuria. Moreover, all of these drugs can cause peptic ulceration. See, Remington's Pharmaceutical Sciences, Mack Pub. Co., Easton, PA (18th ed., 1990) at pages 1115-1122.

There is a large amount of literature on the effect of NSAIDs on cancer, particularly colon cancer. For example, see H. A. Weiss et al., Scand J. Gastroent., 31, 137 (1996) (suppl. 220) and Shiff et al., Exp. Cell Res., 222, 179 (1996). More recently, B. Bellosillo et al., Blood, 92, 1406 (1998) reported that aspirin and salicylate reduced the viability of B-cell CLL cells *in vitro*, but that indomethacin, ketoralac and NS-398, did not. Sulindac has been investigated in combination therapy for the treatment of colon cancer. See, H. M. Verheul et al., Brit. J. Cancer, 79, 114 (1999); F. A. Sinicrope et al., Clin. Cancer Res., 2, 37 (1996); and M. Mooghen et al., J. Pathol., 156, 394 (1988).

C. P. Duffy et al., Eur. J. Cancer, 34, 1250 (1998), reported that the cytotoxicity of certain chemotherapeutic drugs was enhanced when they were combined with certain non-steroidal anti-inflammatory agents. The effects observed against human lung cancer cells and human leukemia cells were highly specific and not predictable; i.e., some combinations of NSAID and agent were effective and some were not. The only conclusion drawn was that the effect was not due to the cyclooxygenase inhibitory activity of the NSAID.

The Duffy group filed a PCT application (WO98/18490) on October 24, 1997, directed to a combination of a "substrate for MRP", which can be an anti-cancer drug, and a NSAID that increases the potency of the anti-cancer drug. NSAIDs recited by the claims are acemetacin, indomethacin, sulindac, sulindac sulfide, sulindac sulfone, tolmetin and zomepirac. Naproxen and piroxicam were reported to be inactive.

Therefore, a continuing need exists for methods to control cancers, and to increase the potency of anti-cancer drugs with relatively non-toxic agents.

Summary of the Invention

In one aspect, the present invention provides a therapeutic method to treat pancreatic cancer, comprising administering to a mammal afflicted with pancreatic cancer an amount of a NSAID, preferably sulindac ((*Z*)-5-fluoro-2-methyl-1-[[4-(methylsulfinyl)phenyl] methylene]-1*H*-Indene-3-acetic acid), or an analog thereof, preferably one that is a COX-2 inhibitor, effective to inhibit the viability of pancreatic cancer cells of said mammal. The present invention also provides a method of increasing the susceptibility of human pancreatic cancer cells to a chemotherapeutic agent comprising contacting the cells with an effective sensitizing amount of a NSAID, preferably sulindac, or said analog thereof. Thus, the invention provides a therapeutic method for the treatment of a human or other mammal afflicted with pancreatic cancer, wherein an effective amount of an NSAID, preferably sulindac or said analog thereof is administered to a subject afflicted with pancreatic cancer and undergoing treatment with a chemotherapeutic ("antineoplastic") agent.

Preferably, sulindac is administered in conjunction with one or more chemotherapeutic agents effective against pancreatic cancer such as gemcitabine or 5-FU.

A method of evaluating the ability of sulindac to sensitize pancreatic cancer cells to a chemotherapeutic agent is also provided. The assay method comprises: (a) isolating a first portion of pancreatic cancer cells from a human cancer patient; (b) measuring their viability; (c) administering sulindac, or said analog thereof, to said patient; (d) isolating a second portion of pancreatic cancer cells from said patient; (e) measuring the viability of the second portion of pancreatic cancer cells; and (f) comparing the viability measured in step (e) with the viability measured in step (b); wherein reduced viability in step (e) indicates that the cells have been sensitized to said chemotherapeutic agent.

Preferably, steps (b) and (e) are carried out in the presence of the chemotherapeutic agent, as will be the case when the pancreatic cancer cells are derived from the blood of a mammal afflicted with pancreatic cancer.

Thus, a cancer patient about to undergo, or undergoing, treatment for pancreatic cancer can be rapidly evaluated to see if he/she will benefit from concurrent chemotherapy and administration of sulindac or an analog thereof.

As used herein, the term "sulindac" and analogs of sulindac includes metabolites such as sulindac sulfone, sulindac sulfide and the pharmaceutically acceptable salts thereof.

Brief Description of the Figures

Figure 1. Photocopy of a representative immunoblot of pancreatic adenocarcinomas and matched normal tissue. Lysates were prepared from tumor (T) specimens obtained from six patients, three with matched normal (N) tissue (sample numbers correspond to those listed in Table I). Lysates were analyzed by immunoblotting with specific COX-2, COX-1, $p21^{ras}$ and actin antibodies as indicated. The positive control (+) for the COX-2 immunoblot is a cell lysate prepared from lipopolysaccharide (LPS)-treated mouse macrophage cell line, Raw 264.7. The negative control (−) is the colon carcinoma cell line HCT 116, which expresses neither COX-1 or COX-2.

Figure 2. Percent COX-2 expression in patient samples. Values of % COX-2 expression for all tumor samples, shown by solid circles, and normal tissue, shown by open circles, from Table I are plotted. Values for mean, median and range are indicated. The % COX-2 expression for the matched pancreatic tumor/normal tissue sets is shown in the inset (n = 11). Lines are drawn between the corresponding tumor values, shown by solid circles, and normal values, shown by the open circles. The difference in COX-2 expression between tumor and normal specimens was determined to be statistically significant (P = 0.004).

Figure 3. COX-2 expression in pancreatic tumor cell lines. A) COX-2 expression in human pancreatic cell lines detected by immunoblot analysis. The K-*ras* mutation status of each of the cell lines is also indicated. B) Activated MAP kinase and COX-2 expression levels following treatment of the cell line BxPC-3 with the MEK inhibitor PD98059 or DMSO for 10 hours. Phosphorylated, active Erk1/2 Map kinase was detected with a phospho-specific Map kinase antibody. C) Comparison of COX-2 expression in three hamster pancreatic cell lines. D27 is the nonmalignant, parental cell line from which the D27/K-*ras* and B12/13 transformed lines were derived. After probing for COX-2, the blot was stripped and re-probed for actin to demonstrate equivalent protein loading. D) Inducibility of COX-2 expression. Serum-starved cells were

stimulated with 10% FCS for the indicated times followed by cell lysis. Expression of COX-2 was determined in lysates prepared from growing (Gr), serum-starved (0 h), and FCS-stimulated cells by immunoblot analysis.

Figure 4. Effect of COX inhibitors on the growth of pancreatic tumor cell lines. The cell lines BxPC-3, shown by the black bars, and PaCa-2, shown by the hatched bars, were plated in the presence of DMSO (control), or the indicated concentrations of sulindac (A), indomethacin (B), or NS-398 (C). On Day 3, cells in duplicate wells were counted and expressed as a percentage of the number of cells growing in the presence of DMSO. The mean +/- SD from at least two independent experiments are shown.

Figure 5. Prostaglandin E_2 production. A) PGE_2 levels in pancreatic tumor cell lines. Following incubation of exponentially growing cells with 15 μM arachidonic acid in serum-free media for one hour, PGE_2 levels in the culture supernatant were determined by enzymeimmunoassay. PGE_2 production was normalized to protein concentration. Results presented are the mean +/- SD from two independent experiments. B) Effect of COX inhibitors on PGE_2 production. The BxPC-3 cell line was incubated with DMSO or two different concentrations of the COX inhibitors (100 μM sulindac, 10 μM indomethacin, and 10 μM NS-398 indicated by the black bars; 250 μM sulindac, 100 μM indomethacin, and 50 μM NS-398 indicated by the stippled bars) for 24 hours prior to cell lysis and measurement of intracellular PGE_2 by enzymeimmunoassay. The % inhibition of PGE_2 by the COX inhibitors is indicated. The data are representative of at least two independent experiments.

Figure 6 is a graph depicting the effect of a combination of sulindac and gemcitabine on the growth of pancreatic tumor cell line BxPC.

Figure 7 is a graph depicting the effect of a combination of sulindac and gemcitabine on the growth of pancreatic tumor cell line PaCa-2.

Detailed Description of the Invention

Difficulty in achieving early diagnosis as well as the aggressive nature of pancreatic cancer contribute to the low survival rate of patients with pancreatic cancer. Since few options exist for the treatment of pancreatic cancer, it is important to identify potential targets for drug therapy. In an effort to gain more

insight into pancreatic tumorigenesis, pancreatic tumors have been analyzed at the molecular level to detect genetic lesions. Activating mutations within the K-*ras* gene have been detected in up to 90% of pancreatic carcinomas, suggesting that activation of the Ras pathway is important in the development of pancreatic cancer (2). Experimental chemotherapeutic strategies for pancreatic cancer patients currently include drugs which target the Ras signal transduction pathway.

Multiple lines of evidence suggest that the enzyme cyclooxygenase (COX), specifically COX-2, may be a promising chemotherapeutic target. For example, epidemiological studies have shown that prolonged use of aspirin or other nonsteroidal anti-inflammatory drugs (NSAIDs) can reduce the risk of colon cancer by 40-50% (3). NSAIDs also inhibit chemically induced colon carcinomas in animal model systems (4). Since NSAIDs are known to inhibit cyclooxygenase (COX), the key enzyme in the conversion of arachidonic acid to prostaglandin and other eicosanoids, these studies imply that COX may play a role in carcinogenesis in addition to its known role in inflammation. Two isoforms of COX, designated COX-1 and COX-2, have been identified. COX-1 is constitutively expressed whereas COX-2 is induced by mitogenic stimuli such as serum, phorbol esters, and growth factors (5, 6). COX-2 expression has recently been shown to be elevated in several different types of human cancer, suggesting that the presence of COX-2 correlates with cancer development (7-11). Additional studies which directly link COX-2 to carcinogenesis include observations that human colon cancer cells expressing COX-2 acquire increased invasiveness (12) and that COX-2 expressed in intestinal epithelial cells inhibits apoptosis (13). COX-2 expression in colon cancer cells has also been found to promote angiogenesis of co-cultured endothelial cells by stimulating the production of angiogenic factors (14). Furthermore, direct genetic evidence linking COX-2 to colorectal tumorigenesis was provided by a mouse model for human familial adenomatous polyposis (FAP), an inherited condition leading to colorectal cancer; in this system, COX-2 gene knockouts and a specific COX-2 inhibitor were found to reduce the number of intestinal polyps formed (15).

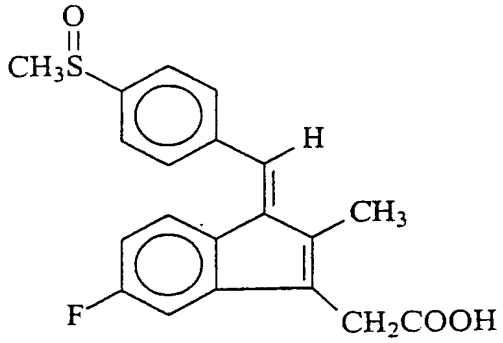
The presence of oncogenic Ras has been associated with the induction of COX-2 expression in H-*ras*-transformed rat intestinal and mammary epithelial

cells as well as in non-small cell lung cancer cell lines (16-18). To our knowledge, the association between oncogenic Ras and COX-2 expression has not ben explored *in vivo*. The high frequency of activating mutations within the K-*ras* gene in pancreatic tumors should enable us to investigate the relationship between oncogenic K-*ras* and COX-2 expression *in vivo*. In the present study, we evaluated COX-2 protein levels in primary human pancreatic adenocarcinomas. We further examined whether COX-2 expression correlated with K-*ras* mutation status in pancreatic tumors as well as in pancreatic cancer cell lines. In light of our data demonstrating elevated levels of COX-2 protein in primary pancreatic tumors and cell lines, we tested the effect of the COX inhibitors sulindac, indomethacin and NS-398 on cell growth and prostaglandin E_2 production in human pancreatic tumor cell lines.

Cyclooxygenase-2 (COX-2) expression is upregulated in several types of human cancers and has also been directly linked to carcinogenesis. To investigate the role of COX-2 in pancreatic cancer, we evaluated COX-2 protein expression in primary human pancreatic adenocarcinomas (n = 23) and matched normal adjacent tissue (n = 11) by immunoblot analysis. COX-2 expression was found to be significantly elevated in the pancreatic tumor specimens compared to normal pancreatic tissue. To examine whether the elevated levels of COX-2 protein observed in pancreatic tumors correlated with the presence of oncogenic K-*ras*, we determined the K-*ras* mutation status in a subset of the tumors and corresponding normal tissues. The presence of oncogenic K-*ras* did not correlate with the level of COX-2 protein expressed in the pancreatic adenocarcinomas analyzed. These observations were also confirmed in a panel of human pancreatic tumor cell lines. Furthermore, in the pancreatic tumor cell line expressing the highest level of COX-2 (BxPC-3), COX-2 expression was demonstrated to be independent of Erk1/2 Map kinase activation. The lack of correlation between COX-2 and oncogenic K-*ras* expression suggests that Ras activation may not be sufficient to inducing COX-2 expression in pancreatic tumor cells and that the aberrant activation of signaling pathways other than Ras may be required for up-regulating COX-2 expression. We also report that the COX inhibitors sulindac, indomethacin, and NS-398 inhibited cell growth in both COX-2-positive (BxPC-3) and COX-2-negative (PaCa-2) pancreatic tumor

cell lines. However, suppression of cell growth by indomethacin and NS-398 was significantly greater in the BxPC-3 cell line compared to the PaCa-2 cell line ($P = 0.004$ and $P < 0.001$ respectively). In addition, the three COX inhibitors reduced prostaglandin E_2 (PGE_2) levels in the BxPC-3 cell line. Taken together, our data suggest that COX-2 may play an important role in pancreatic tumorigenesis and therefore be a promising chemotherapeutic target for the treatment of pancreatic cancer.

Sulindac ((Z)-5-fluoro-2-methyl-1-[[4-(methylsulfinyl)phenyl] methylene]-1*H*-Indene-3-acetic acid) has the formula:

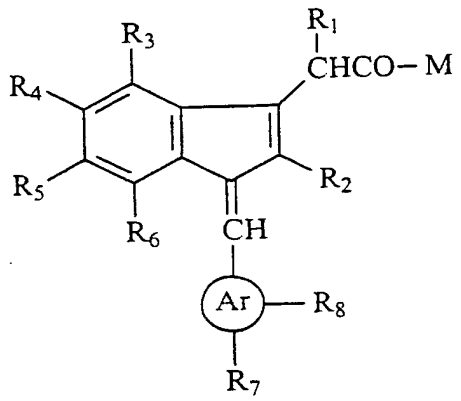


and is available from Merck as Clinoril®.

Sulindac is an indene-type anti-inflammatory agent indicated for acute and long-term relief of signs and symptoms of osteoarthritis, rheumatoid arthritis, ankylosing spondylitis, acute painful shoulder and acute gouty arthritis. It also possesses analgesic and anti-pyretic properties. Its precise mechanism of action is unknown; however, it is thought the sulfide metabolite may inhibit prostaglandin synthesis. It is absorbed approximately 90% after oral administration. Peak plasma levels are achieved in about 2 hr in the fasting patient and 3 to 4 hr when administered with food. The mean half-life of sulindac is 7.8 hr; the mean half-life of the sulfide metabolite is 16.4 hr. It is contraindicated in functional Class IV arthritis (incapacitated, largely or wholly bedridden, or confined to a wheelchair; little or no self-care), patients in whom acute asthmatic attacks, urticaria or rhinitis are precipitated by aspirin or other nonsteroidal anti-inflammatory agents and in patients sensitive to the drug.

Its synthesis and analogs thereof (including metabolites and salts) are disclosed in, e.g., U.S. Pat. Nos. 3,654,349 and 3,647,858, and in Fed. Proc., 31, 377 (1972); Drug. Metab. Disps., 1, 721 (1972).

For example, analogs of sulindac include substituted indenyl acetic acids of the formula (I):



(I)

wherein:

(Ar) may be aryl or heteroaryl, e.g., a 5-, 6- or 7-membered hetroaryl ring comprising 1-3, N, O and/or S atoms;

R_1 may be hydrogen, lower(C_1-C_4)alkyl or halogenated lower-alkyl;

R_2 may be hydrogen or (C_1-C_6)alkyl;

R_3, R_4, R_5 and R_6 each may be hydrogen, alkyl, (C_2-C_5)acyloxy, alkoxy, nitro, amino, acylamino, alkylamino, dialkylamino, dialkylaminoalkyl, sufamyl, alkythio, mercapto, hydroxy, hydroxyalkyl, alkylsulfonyl, halogen, cyano, carboxyl, carbalkoxy, carbamido, halogenoalkyl, cycloalkyl or cycloalkoxy;

R_7 may be alkylsulfinyl or alkylsulfonyl;

R_8 may be hydrogen, halogen, hydroxy, alkoxy, or haloalkyl; and

M may be hydroxy, loweralkoxy, substituted loweralkoxy, amino, alkylamino, dialkylamino, N-morpholino, hydroxyalkylamino, polyhydroxyalkylamino, dialkylaminoalkylamino, aminoalkylamino, and the group OMe, in which Me is a cation; and pharmaceutically acceptable salts thereof.

Alkyl is preferably lower alkyl, i.e., (C_1 - C_4) alkyl. Acyl is preferably (C_2 - C_4) acyl.

Other NSAIDs, including indomethacin and NS-398 also inhibit the growth of pancreatic tumor cell lines, as discussed hereinbelow, and can also be used in the present method, alone, or preferably in combination with sulindac.

Gemcitabine (Gemcitabine-HCl or Gemzar®) is a 2′-deoxy-2′, 2′-difluorocytidine-HCl (B-isomer), an antitumor nucleoside analog. It can be administered by injection or infusion in dosages of about 500-4000 mg/m^2/week for up to 7 weeks/cycle for treatment of localized or metastatic pancreatic cancer (adenocarcinoma of the pancreas). It can also be administered in conjunction with other anti-cancer agents, such as 5-FU. See, PDR (53rd ed., 1999) at pages 1578-1582.

The effect of sulindac or NS-398 alone and in combination with gemcitabine on the growth of pancreatic tumor cells BxPC-3 and PaCa-2 was investigated. Treatment with the drug combinations inhibited the growth of both cell lines to a greater extent than did either compound alone. Parthenolide, the active ingredient from feverfew with anti-inflammatory properties, was also found to inhibit the growth of PaCa-2 cells and in combination with gemcitabine exhibited greater inhibitory effects than either compound alone. NF-κB DNA binding activity was inhibited by parthenolide treatment. These results suggest that anti-inflammatory drugs may enhance the effectiveness of gemcitabine against pancreatic tumors.

The magnitude of a prophylactic or therapeutic dose of sulindac, an analog thereof or a combination thereof, in the acute or chronic management of cancer, i.e., pancreatic caner, will vary with the stage of the cancer, such as the solid tumor to be treated, the chemotherapeutic agent(s) or other anti-cancer therapy used, and the route of administration. The dose, and perhaps the dose frequency, will also vary according to the age, body weight, and response of the individual patient. In general, the total daily dose range for sulindac and its analogs, for the conditions described herein, is from about 50 mg to about 2500 mg, in single or divided doses. Preferably, a daily dose range should be about 100 mg to about 1500 mg, in single or divided doses, most preferably about 150-500 mg per day. In managing the patient, the therapy should be initiated at a lower dose and increased depending on the patient's global response. It is further recommended that infants, children, patients over 65 years, and those

with impaired renal or hepatic function initially receive lower doses, and that they be titrated based on global response and blood level. It may be necessary to use dosages outside these ranges in some cases. Further, it is noted that the clinician or treating physician will know how and when to interrupt, adjust or terminate therapy in conjunction with individual patient response. The terms "an effective amount" or "an effective sensitizing amount" are encompassed by the above-described dosage amounts and dose frequency schedule.

Any suitable route of administration may be employed for providing the patient with an effective dosage of sulindac. For example, oral, rectal, parenteral (subcutaneous, intravenous, intramuscular), intrathecal, transdermal, and like forms of administration may be employed. Dosage forms include tablets, troches, dispersions, suspensions, solutions, capsules, patches, and the like. The sulindac may be administered prior to, concurrently with, or after administration of chemotherapy, or continuously, i.e., in daily doses, during all or part of, a chemotherapy regimen. The sulindac, in some cases, may be combined with the same carrier or vehicle used to deliver the anti-cancer chemotherapeutic agent.

Thus, the present compounds may be systemically administered, e.g., orally, in combination with a pharmaceutically acceptable vehicle such as an inert diluent or an assimilable edible carrier. They may be enclosed in hard or soft shell gelatin capsules, may be compressed into tablets, or may be incorporated directly with the food of the patient's diet. For oral therapeutic administration, the active compound may be combined with one or more excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 0.1% of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 2 to about 60% of the weight of a given unit dosage form. The amount of active compound in such therapeutically useful compositions is such that an effective dosage level will be obtained.

The tablets, troches, pills, capsules, and the like may also contain the following: binders such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrated agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium

stearate; and a sweetening agent such as sucrose, fructose, lactose or aspartame or a flavoring agent such as peppermint, oil of wintergreen, or cherry flavoring may be added. When the unit dosage form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier, such as a vegetable oil or a polyethylene glycol. Various other materials may be present as coatings or to otherwise modify the physical form of the solid unit dosage form. For instance, tablets, pills, or capsules may be coated with gelatin, wax, shellac or sugar and the like. A syrup or elixir may contain the active compound, sucrose or fructose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavoring such as cherry or orange flavor. Of course, any material used in preparing any unit dosage form should be pharmaceutically acceptable and substantially non-toxic in the amounts employed. In addition, the active compound may be incorporated into sustained-release preparations and devices.

The active compound may also be administered intravenously or intraperitoneally by infusion or injection. Solutions of the active compound or its salts can be prepared in water, optionally mixed with a non-toxic surfactant. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, triacetin, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical dosage forms suitable for injection or infusion can include sterile aqueous solutions or dispersions or sterile powders comprising the active ingredient which are adapted for the extemporaneous preparation of sterile injectable or infusible solutions or dispersions, optionally encapsulated in liposomes. In all cases, the ultimate dosage form must be sterile, fluid and stable under the conditions of manufacture and storage. The liquid carrier or vehicle can be a solvent or liquid dispersion medium comprising, for example, water, ethanol, a polyol (for example, glycerol, propylene glycol, liquid polyethylene glycols, and the like), vegetable oils, non-toxic glyceryl esters, and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the formation of liposomes, by the maintenance of the required particle size in the case of dispersions or by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal

agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, buffers or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compound in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filter sterilization. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze drying techniques, which yield a powder of the active ingredient plus any additional desired ingredient present in the previously sterile-filtered solutions.

Useful dosages of the compounds of sulindac and its analogs can be determined by comparing their *in vitro* activity, and *in vivo* activity in animal models. Methods for the extrapolation of effective dosages in mice, and other animals, to humans are known to the art; for example, see U.S. Patent No. 4,938,949.

The invention will be described by reference to the following detailed examples, in which the following materials and methods were employed:

1. Patient Samples. Banked tissues were obtained from the Indiana University Tissue Procurement Laboratory and the Cooperative Human Tissue Network (CHTN) which is funded by the National Cancer Institute. A total of 23 primary human pancreatic cancer specimens were analyzed in this study. Corresponding matched, normal adjacent tissue was obtained from 11 of the patients. The patients were selected on the basis of having no prior chemotherapy. Tissues were frozen in liquid nitrogen within 1 hour of surgical removal and subsequently stored at -80°C. Paraffin sections were prepared from a subset of the specimens. All tumor specimens used in this study were examined by a pathologist and classified as primary pancreatic adenocarcinomas. Institutional Review Board (IRB) approval was obtained for this study.

2. Immunoblots. Frozen tissue was briefly homogenized in RIPA lysis buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1 mM Na_3VO_4, 20 mM β-glycerophosphate, 1 mM sodium fluoride, pepstatin

(1 μg/ml), aprotinin (20 μg/ml), and leupeptin (1 μg/ml). Lysates were clarified by centrifugation at 13,000 × g for 10 minutes, followed by boiling in sample buffer. Protein concentrations were determined using the BCA protein assay kit (Pierce). Equivalent amounts of total protein were resolved by SDS-PAGE on 10% gels (Novex) and transferred to Immobilon P membranes (MSI). The blots were probed with the primary antibodies COX-1 (C-20, Santa Cruz Biotechnology), COX-2 (C-20, Santa Cruz Biotechnology), $p21^{ras}$ (pan-ras Ab-3, Oncogene Science), actin (C-11, which recognizes a broad range of actin isoforms, Santa Cruz Biotechnology) or the phospho-specific Map kinase antibody (New England Biolabs) as recommended by the manufacturers. Bands were visualized by ECL (Amersham) and quantitated by densitometry. The amount of COX-2 was expressed as a percentage of the positive control (3 μg of cell lysate prepared from the mouse macrophage cell line Raw 264.7 stimulated with lipopolysaccharide, LPS). Specific recognition of COX-1 and COX-2 respectively by the COX-1 and COX-2 antibodies was confirmed by peptide inhibition experiments (data not shown).

3. Immunohistochemistry. Immunohistochemical stains were performed on 5 μm formalin-fixed, paraffin-embedded sections using the avidin-biotin-complex technique (19). Primary polyclonal antibody was used for evaluation of COX-2 expression (Oxford Biomedical Research, Inc., Oxford, MI; 1:100 dilution). 3,3′-diaminobenzidine (DAB) was used as the chromogen and 0.2% methyl green was used as the counterstain. Positive and negative controls were run in parallel and gave appropriate results.

4. K-*ras* mutation analysis. Genomic DNA was prepared by incubating the tissue in lysis buffer (50 mM Tris pH 8.0, 100 mM EDTA, 100 mM NaCl, 1% SDS, 0.5 mg/ml proteinase K) overnight at 55°C. RNAse (0.1 mg/ml) was added and the incubation was continued for 2 hours at 37°C. The sample was then extracted with phenol, phenol:chloroform, and ethanol precipitated. K-*ras* exon 1 of the resuspended genomic DNA (0.5 μg) was amplified by PCR (5′ primer = 5′-ATGACTGAATATAAACTTGT-3′ (SEQ ID NO:1); 3′ primer = 5′-CTCTATTGTTGGATCATATT-3′) (SEQ ID NO:2) (20). K-*ras* mutation specific oligonucleotides (Oncogene Research Products) were utilized to detect mutations at K-*ras* codon 12 in the PCR-amplified products by dot blot

hybridization (21). Mutations at K-*ras* codon 13 were detected by sequencing the purified PCR amplification products.

5. Statistical Analysis. The presence of statistically significant elevation of COX-2 protein between cancer specimens and corresponding normal adjacent tissues was determined by the nonparametric signed rank test. A two-way analysis of variance (ANOVA) was used to examine the difference in mean percent cell growth between the BxPC-3 and PaCa-2 cell lines in the presence of the COX inhibitors.

6. Cell Lines. The human pancreatic tumor cell lines (AsPC-1, BxPC-3, Capan-1, Capan-2, HPAF-II, Hs766T, PaCa-2 and PANC-1) were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured as recommended. The hamster pancreatic cell lines (D27, D27/K-*ras*, B12/13) were cultured as described previously (22, 23). For the inhibitor studies, BxPC-3 cells were treated with the Map kinase kinase (MEK) inhibitor PD98059 (40 μM) or DMSO for 10 hours. Lysates were prepared and analyzed as described above.

7. Cell Growth. Cells were plated in duplicate in 6-well plates in the presence of DMSO, sulindac (Sigma), indomethacin (Sigma), or NS-398 (Biomol). On Day 3, cells were trypsinized, stained with trypan blue and counted using a hemocytometer. Cell growth was determined by averaging the cell counts and expressed as a percentage of the number of cells in the DMSO control samples.

8. Prostaglandin E_2 Assay. Cells were plated in 12-well plates. On Day 3, the culture medium was aspirated and replaced with 15 μM arachidonic acid in serum-free media for one hour prior to assaying the culture supernatant for PGE_2 by enzymeimmunoassay (Biotrak, Amersham) as recommended by the manufacturer. The amount of PGE_2 produced was normalized to protein concentration. Intracellular PGE_2 levels were determined by plating cells in 12-well plates in the presence of the COX inhibitors for 24 hours followed by cell lysis and quantitation by enzymeimmunoassay (Biotrak) as recommended by the manufacturer. For the determination of intracellular PGE_2 levels, the cells were not preincubated with arachidonic acid prior to assay.

9. Abbreviations. COX, cyclooxygenase; Erk 1/2 MAP kinase (Erk, MAPK), extracellular signal-regulated kinase 1 and 2 mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; NSAIDS, nonsteroidal anti-inflammatory drugs; PGE_2, prostaglandin E_2, PMA, phorbol 12-myristate 13-acetate.

Example 1

COX-2 expression in human pancreatic adenocarcinomas

The expression of COX-2 protein was examined in primary human pancreatic adenocarcinomas (n = 23) and matched normal adjacent pancreatic tissue (n = 11) by immunoblot analysis. A representative immunoblot of lysates prepared from six patients, three with matched normal tissue lysates, is shown in Figure 1. Undetectable levels of COX-2 protein were observed in each of the normal specimens. In contrast, COX-2 protein expression in the pancreatic tumor tissues ranged from undetectable (sample #21) to slight/moderate (samples #12, 14, 20) to high levels (samples #9, 22). COX-1 protein was observed in both pancreatic tumor and normal tissues, although the level of expression was variable and not consistently elevated in the tumor specimens (Figure 1). Similar levels of $p21^{ras}$ and actin expression were found in both the tumor and corresponding normal tissues (Figure 1).

The percent of COX-2 expression was determined for all the tissue specimens by performing densitometic analysis and calculated relative to the positive control set equal to 100% (Table I and graphically in Figure 2). The positive control for our studies was the mouse macrophage cell line Raw 264.7 stimulated with lipopolysaccharide (LPS), previously shown to induce COX-2 expression (24). A wide range (0-93%) of COX-2 expression was found in the pancreatic adenocarcinomas versus a much narrower range (0-4.3%) of COX-2 expression in the normal tissues. Both the mean and median COX-2 expression were higher in the tumor samples, suggesting that COX-2 expression is elevated in pancreatic adenocarcinomas compared to normal tissue. The difference in COX-2 expression between the pancreatic tumor and corresponding normal tissue was determined to be statistically significant (P = 0.004) (Figure 2, inset). COX-2 positive and negative samples were defined as those samples with

percent COX-2 expression greater or less than 5% respectively, which corresponds closely with visual detection in the immunoblots. According to these criteria, 6 out of 11 (55%) tumor samples in the matched tissue sets were COX-2 positive. Similarly, 13 out of the 23 (56%) total tumor specimens analyzed were COX-2 positive; in contrast, all the normal tissue samples (n = 11) were COX-2 negative.

Immunohistochemical staining of the pancreatic tumor specimens demonstrated that COX-2 expression was localized to the carcinoma cells and was not detectable in the stromal compartment of the tumors (Figure 3).

Example 2

COX-2 expression and K-*ras* mutation in pancreatic tumors and cell lines

To determine if COX-2 expression levels correlated with the K-*ras* mutation status of the tumors, genomic DNA was isolated from a subset of the tissue specimens and screened for the presence of K-*ras* mutations at codon 12 by allele-specific hybridization of PCR-amplified K-*ras* exon 1 products. Specimens which lacked a mutation at codon 12 were subsequently sequenced to search for the presence of K-*ras* mutations at codon 13 (Table I). All of the normal tissues analyzed were wild-type at codon 12 (GGT = Gly) and codon 13 (GGC = Gly). Of the 13 pancreatic cancer specimens analyzed, one specimen had a mutation at codon 13 whereas 10 samples were mutated at codon 12, corresponding to a K-*ras* mutation frequency of 85%. There did not appear to be a direct correlation between K-*ras* mutation and the extent of COX-2 protein expression. For example, some samples expressed high levels of COX-2 protein and possessed a mutation in K-*ras* (i.e., tumor samples #9, 16 and 22); however, other samples which had mutated K-*ras* expressed little or no COX-2 protein (i.e., tumor samples #3, 17, 18, 19, and 21).

Similarly, no direct correlation between the expression of activated K-*ras* and COX-2 was observed in a panel of human pancreatic adenocarcinoma cell lines with known K-*ras* mutation status (25, 26). Both the frequency and variability in the quantity of COX-2 expressed in the pancreatic tumor cell lines reflected our findings in the primary pancreatic adenocarcinomas. Of the eight human pancreatic tumor cell lines analyzed, only three of the seven cell lines

expressing oncogenic K-*ras* exhibited detectable levels of COX-2 protein (Capan-1, Capan-2 and HPAF-II) (Figure 4A). High levels of COX-2 protein were also observed in the wild-type K-*ras*-expressing cell line BxPC-3, which displays high levels of Ras-independent Raf activity (26). Treatment of the BxPC-3 cell line with the Map kinase kinase (MEK) inhibitor PD98059 significantly reduced the level of active, phosphorylated Erk1/2 Map kinase but had no effect on the amount of COX-2 protein synthesized, suggesting that COX-2 expression is independent of Erk1/2 activation (Figure 4B). Taken together, our results suggest that activation of the Ras pathway is not sufficient for mediating COX-2 upregulation in pancreatic tumor cells. We also compared the level of COX-2 expression in three hamster pancreatic cell lines. The D27/K-*ras* and B12/13 transformed cell lines were derived from the nonmalignant, hamster pancreatic duct cell line, D27, by transfection with oncogenic K-*ras* or treatment with chemical carcinogens *in vitro* respectively (22, 23). Although both the D27/K-*ras* and B12/13 cell lines harbor oncogenic K-*ras*, only the B12/13 cells showed elevated levels of COX-2 protein compared to the D27 parental line (Figure 4C). These results confirm our conclusion that Ras activation alone is not sufficient for upregulating COX-2 expression in pancreatic cancer cells and suggest that additional events which occur following exposure to chemical carcinogens may be required.

To examine whether COX-2 expression could be induced in the human pancreatic cancer cell lines, four cell lines were serum-starved and subsequently treated with 10% FCS for various time periods (Figure 4D). In the COX-2-positive BXPC-3 and Capan-1 cell lines, COX-2 was still detectable following serum starvation, although at lower levels than in exponentially growing cells. COX-2 expression was inducible in these cell lines following FCS stimulation. In contrast, COX-2 expression could not be induced by serum treatment in either of the COX-2-negative cell lines, AsPC-1 and PaCa-2. Under these treatment conditions, Erk1/2 is activated (unpublished observations), again demonstrating that Erk 1/2 activation is not sufficient for inducing COX-2 expression in the COX-2-negative pancreatic tumor cells. We observed similar results upon treating the cell lines with the tumor promoter, PMA (unpublished observations).

Example 3

Treatment of pancreatic tumor cell lines with cyclooxygenase inhibitors

The COX-2-positive human pancreatic tumor cell lines, BxPC-3, and the COX-2-negative cell line, PaCa-2, were treated with the COX inhibitors sulindac, indomethacin, or NS-398. Sulindac and indomethacin are nonselective COX inhibitors, inhibiting both COX-1 and COX-2 (27); whereas NS-398 is a more specific inhibitor of COX-2 (28). The effect of the COX inhibitors on cell growth was measured after three days of treatment (Figure 5). All three inhibitors were found to suppress cell growth in both pancreatic tumor cell lines in a dose-dependent manner. However, indomethacin and NS-398 were found to inhibit cell growth to a greater extent in the COX-2 expressing cell line BxPC-3 compared to the PaCa-2 cell line ($P = 0.004$ and $P < 0.001$ respectively). No significant difference in cell growth inhibition was observed between the two cell lines with sulindac treatment ($P = 0.333$).

To evaluate the functional activity of COX-2 in the human pancreatic tumor cell lines, prostaglandin E_2 (PGE_2) production was measured by enzymeimmunoassay (Figure 6A). PGE_2 production was elevated in the BxPC-3, Capan-1, Capan-2 and HPAF-II cell lines, correlating with the increased level of COX-2 expressed in these cell lines. In contrast, barely detectable levels of PGE_2 were detected in the COX-2-negative pancreatic cell lines. To determine the effect of the COX inhibitors on PGE_2 production, the COX-2-positive cell line BxPC-3 was incubated with two different concentrations of sulindac (100, 250 μM), indomethacin (10, 100 μM), or NS-398 (10, 50 μM) for 24 hours prior to measuring intracellular PGE_2 levels (Figure 6B). Intracellular PGE_2 rather than PGE_2 secreted into the growth medium was measured since intracellular levels should be more sensitive to changes in response to the inhibitor treatments. The two concentrations of the various COX inhibitors were evaluated in the cell growth assays described above (Figure 5), with the higher concentration corresponding to the IC_{50} values of the COX inhibitors. At the lower concentrations, both NS-398 and indomethacin inhibited PGE_2 production, 75% and 95% respectively; in contrast, sulindac at the lower concentration did not affect PgE_2 levels. Nevertheless, all three inhibitors at the higher IC_{50} concentrations substantially suppressed intracellular PGE_2 levels, between 86%

to 98%, in the BxPC-3 cell line. These data suggest that the COX inhibitors may exert their effects in the COX-2 positive BxPC-3 cell line at least in part by decreasing PGE_2 production. However, in the COX-2 negative cell lines, the effects of the COX inhibitors may be mediated by COX- and PGE_2-independent mechanisms.

Example 4

Synergistic effect of combinations of sulindac and gemcitabine

BxPC-3 or PaCa-2 cells were plated on day 0. The compounds sulindac (sul, microM), gemcitabine (Gem, nM), or the combination as indicated in figures 6-7 were added on the following day (day 1). Cells were trypsinized and counted manually on day 3. Each point was set up in duplicate for each experiment. Cell number is expressed as % survival relative to the number of cells present in the media + solvent control sample, set to 100%. Percent survival for sulindac alone samples is also shown (Gem, 0 nM).

These data demonstrate that the combination of sulindac and gemcitabine is more effective than either compound alone in pancreatic tumor cells.

In resting cells, the expression of COX-2 is usually undetectable but can be rapidly induced by mitogenic stimuli as well as inflammatory agents (5, 6, 29). Recent studies have shown that COX-2 expression is upregulated in a variety of human cancers, including colon, lung, gastric, pancreatic and esophageal (7-11). In the present study, we report that elevated levels of COX-2 protein are expressed in human pancreatic tumors compared to barely detectable levels in the matched normal pancreatic tissue, suggesting that increased expression of COX-2 protein correlates with pancreatic tumorigenesis. Our results confirm a recent report demonstrating upregulation of COX-2 RNA and protein in pancreatic tumors and localization of COX-2 in malignant epithelial cells (11). An earlier study demonstrated that the expression of group II phospholipase A2, which catalyzes the release of arachidonic acid from membrane phospholipids, was higher in pancreatic ductal adenocarcinomas compared to normal pancreatic tissue (30). In addition, the development of *N*-nitrosobis(2-oxopropyl)amine (BOP)-initiated pancreatic tumors in hamsters was inhibited by the administration of two prostaglandin synthesis inhibitors,

phenylbutazone and indomethacin (31). Together with our observations *in vivo* and *in vitro*, these studies suggest that increased prostaglandin production due to the increased expression of COX-2 may be an important event in the multi-step progression towards pancreatic tumor formation.

Recent studies have suggested that Ras activation may induce COX-2 expression in several systems. Activated, oncogenic H-*ras* was inducibly expressed in Rat-1 fibroblasts with a concomitant increase in COX-2 expression as well as PGE_2 production (32). In this particular cell line, a specific mitogen-activated protein kinase kinase (MEK) inhibitor, PD98059, was found to suppress COX-2 induction by oncogenic Ras, suggesting that Erk1/2 activation is required for Ras-dependent induction of COX-2. Similarly, elevated levels of COX-2 as well as prostaglandin E_2 were detected in Ras-transformed mammary epithelial cells (C57/MG) cells (17). In human non-small cell lung cancer (NSCLC) cell lines expressing oncogenic K-Ras, increased PGE_2 production was mediated by constitutively high expression of cytosolic phospholipase A_2 and COX-2 compared with NSCLC lines without Ras mutations (18). In the present study, we addressed the question of whether the expression of oncogenic K-Ras correlated with increased COX-2 expression in primary human pancreatic adenocarcinomas. We found that the presence of a mutation at codons 12 or 13 in the K-*ras* gene did not correlate with the expression of detectable levels of COX-2 protein. A possible explanation for the lack of COX-2 expression in a subset of the tumors with oncogenic Ras is that Erk1/2 activity may be down-regulated in pancreatic carcinomas (26). Moreover, even in the two pancreatic tumor samples which did show elevated levels of activated Erk1/2 (samples #4 and 21, data not shown), only low levels of COX-2 were detected in the present study, suggesting that Erk1/2 activation alone is not sufficient for inducing COX-2 expression. These findings suggest that within the tumor environment, the presence of oncogenic K-*ras* does not directly result in increased COX-2 expression in pancreatic cancer.

Similar conclusions were also reached upon analysis of pancreatic cancer cell lines, which were examined since they represent a homogenous population of cells as opposed to primary tumor tissue which is heterogenous. Despite activating *K-ras* mutations in seven out of the eight lines, only three of the lines

with mutated *K-ras* expressed detectable amounts of COX-2 protein. COX-2 expression was also elevated in the wild-type K-*ras* BxPC-3 cell line, which possesses high levels of active Raf (26). COX-2 was constitutively expressed in the four COX-2-positive cell lines; in contrast COX-2 expression could not be induced by serum or PMA in the COX-2-negative cell lines, suggesting that COX-2 expression is blocked in these cells. COX-2 expression in the BxPC-3 cell line was not reduced following treatment with the MEK inhibitor PD98059, suggesting that COX-2 induction is independent of Ras pathway activation in this cell line. Differential expression of COX-2 protein in the transformed hamster pancreatic cell lines generated by either transfection with oncogenic Ras or treatment with chemical carcinogens further demonstrated that Ras activation does not appear to be sufficient for mediating the induction of COX-2 expression. Activation of other signaling pathways in addition to Ras may cooperate to determine the extent of COX-2 expression in cancer cells. Such pathways may include the p38 mitogen-activated protein kinase which has been reported to regulate the induction of COX-2 in lipopolysaccharide-treated human monocytes (33). Furthermore, in human vascular endothelial cells, the NF-κB p65 transcription factor was found to mediate the induction of COX-2 in response to hypoxia (34). Thus, the induction of COX-2 expression appears to be mediated by multiple signaling pathways. The specific pathway(s) activated may depend upon the cell type as well as the stimulus. Further experiments will be required to delineate which signaling pathways are function in pancreatic tumor cells.

Cell growth was inhibited by treatment with the COX inhibitors sulindac, indomethacin or NS-398 in both COX-2-positive (BxPC-3) and -negative (PaCa-2) cell lines. However, the cell line BxPC-3 was significantly more sensitive to growth inhibition by indomethacin and NS-398 compared to the PaCa-2 cell line, suggesting that these two compounds may be more selective for COX-2 expressing cells than sulindac. Furthermore, in the BxPC-3 cell line, the COX inhibitors at IC_{50} concentrations substantially decreased intracellular PGE_2 levels. No PGE_2 was detectable in the non-COX-2 expressing cell lines. These data suggest that the COX inhibitors exert their inhibitory effects by both COX/PGE_2-dependent and -independent pathways in pancreatic tumor cell lines.

NS-398 has been previously shown to inhibit cell proliferation by inducing apoptosis in a COX-2 independent fashion (35). Similarly, sulindac sulfone, a metabolite of sulindac which does not inhibit COX activity, was found to inhibit colon carcinogenesis in a rat model without decreasing prostaglandin levels (36). Furthermore, in cyclooxygenase null embryo fibroblasts, transformation as well as the antiproliferative and antineoplastic actions of NSAIDs were recently were recently shown to be independent of COX expression (37).

The detection of elevated levels of COX-2 in a variety of human cancers combined with the chemopreventative effect of NSAIDs in colon cancer demonstrate that COX-2 is an important participant in carcinogenesis. The reported biological consequences of COX-2 upregulation include inhibition of apoptosis (13), increased metastatic potential (12) and promotion of angiogenesis (14). These events may contribute to cell transformation and tumor progression. COX-2 expression was noticeably elevated in 55% of the patient pancreatic tumor samples analyzed, identifying COX-2 as a new target for chemotherapy. These results demonstrating the ability of COX inhibitors to inhibit pancreatic tumor cell growth and PGE_2 production *in vitro* indicate that NSAIDs may be effective in the treatment of pancreatic cancer patients, for whom few treatment options currently exist. COX-2 expression is also useful as a prognostic or diagnostic tool.

REFERENCES:

1. Landis et al., CA Cancer J. Clin., 48, 6-29 (1998).
2. Almoguera et al., Cell, 53, 549–54 (1988).
3. Thun, M.J., Cancer Metastasis Rev., 13, 269-77 (1994).
4. Giardiello et al., Eur. J. Cancer, 31A, 1071-6 (1995).
5. DeWitt et al., Arch. Biochem. Biophys., 306, 94-102 (1993).
6. Hamasaki et al., Arch. Biochem. Biophys., 304, 226-34 (1993).
7. Eberhart et al., Gastroenterology, 107, 1183-8 (1994).
8. Ristimaki et al., Cancer Res., 57, 1276-80 (1997).
9. Zimmermann et al., Cancer Res., 59, 198-204 (1999).
10. Wolff et al., Cancer Res., 58, 4997-5001 (1998).
11. Tucker et al., Cancer Res., 59, 987-90 (1999).

12. Tsujii et al., Proc. Natl. Acad. Sci. USA, 94, 3336-40 (1997).
13. Tsujii et al., Cell, 83, 493-501 (1995).
14. Tsujji et al., Cell, 93, 705-16 (1998).
15. Oshima et al., Cell, 87, 803-9 (1996).
16. Sheng et al., Gastroenterology, 113, 1883-91 (1997).
17. Subbaramaiah et al., Cancer Res., 56, 4424-9 (1996).
18. Heasley et al., J. Biol. Chem., 272, 14501-4 (1997).
19. Hsu et al., J. Histochem. Cytochem., 29, 577-80 (1981).
20. Sumi et al., Pancreas, 9, 657-61 (1994).
21. Konishi et al., Cancer, 69, 2293-99 (1992).
22. Mangold et al., Carcinogenesis, 15, 1979-84 (1994).
23. Stayrook et al., Anticancer Res., 18, 823-8 (1998).
24. Riese et al., J. Leukoc. Biol., 55, 476-82 (1994).
25. Berrozpe et al., Int. J. Cancer, 58, 185-91 (1994).
26. Yip-Schneider et al., Int. J. Oncol., 15, 271-9 (1999).
27. Meade et al., J. Biol. Chem., 268, 6610-4 (1993).
28. Futaki et al., Prostaglandins, 47, 55-9 (1994).
29. Lee et al., J. Biol. Chem., 267, 25934-8 (1992).
30. Kiyohara et al., Int. J. Pancreatol., 13, 49-57 (1993).
31. Takahashi et al., Carcinogenesis, 11, 393-5 (1990).
32. Sheng et al., J. Biol. Chem., 273, 22120-7 (1998).
33. Dean et al., J. Biol. Chem., 274, 264-9 (1999).
34. Schmedtje et al., J. Biol. Chem., 272, 601-8 (1997).
35. Elder et al., Clin. Cancer Res., 3, 1679-83 (1997).
36. Piazza et al., Cancer Res., 57, 2909-15 (1997).
37. Zhang et al., J. Exp. Med., 190, 451-59 (1999).

TABLE I. Analysis of Patient Samples

Tissue Sample[a]	Tissue Type	% COX-2[b]	% Cancer[c]	K-ras Mutation[d]
1	pancreatic adenocarcinoma	7.0	10	WT = GGT (Gly)
2	pancreatic adenocarcinoma	2.0	95	ND
3	pancreatic adenocarcinoma	0.2	15	GGC to CGC (Arg, codon 13)
4	pancreatic adenocarcinoma	3.6	35	GGT to GAT (Asp)
4N	normal	0.2	–	GGT
5	pancreatic adenocarcinoma	5.2	30	GGT
6	pancreatic adenocarcinoma	0	ND	ND
7	pancreatic adenocarcinoma	5.9	ND	ND
7N	normal	0	–	ND
8	pancreatic adenocarcinoma	3.7	ND	ND
9	pancreatic adenocarcinoma	93	ND	GGT to GAT (Asp)
10	pancreatic adenocarcinoma	4.8	ND	ND
11	pancreatic adenocarcinoma	14.2	40	ND
11N	normal	0.1	–	ND
12	pancreatic adenocarcinoma	16	15	ND
14	pancreatic adenocarcinoma	31	ND	ND

Tissue Sample[a]	Tissue Type	% COX-2[b]	% Cancer[c]	K-ras Mutation[d]
15	pancreatic adenocarcinoma	7.8	25	GGT to GAT (Asp)
15N	normal	4.3	–	GGT
16	pancreatic adenocarcinoma	66	35	GGT to GAT (Asp)
16N	normal	0	–	GGT
17	pancreatic adenocarcinoma	0.1	40	GGT to GTT (Val)
17N	normal	0.1	–	GGT
18	pancreatic adenocarcinoma	1.1	40	GGT to GAT (Asp)
18N	normal	0	–	GGT
19	pancreatic adenocarcinoma	2.2	95	GGT to GAT (Asp)
19N	normal	1.1	–	GGT
20	pancreatic adenocarcinoma	7.9	30	GGT to CGT (Arg), TGT (Cys)
20N	normal	1.9	–	GGT
21	pancreatic adenocarcinoma	0	20	GGT to CGT (Agr), TGT (Cys)
21N	normal	0.7	–	GGT
22	pancreatic adenocarcinoma	63.9	15	GGT to GAT (Asp)
22N	normal	0.7	–	GGT
23	pancreatic adenocarcinoma	15	20	ND
24	pancreatic adenocarcinoma	0	ND	ND

[a] A total of 23 pancreatic adenocarcinomas were obtained and numbered as indicated. Matched, normal adjacent tissue (N) was also obtained from 11 patients.

[b] The percent COX-2 expression was determined by performing densitometric analysis of COX-2 immunoblots and expressed relative to the positive control set equal to 100%.

[c] The percent cancer was determined by visualization following hematoxylin/eosin staining of slides prepared from paraffin sections.

[d] K-*ras* mutation status at codon 12 was determined by allele-specific hybridization of K-*ras* exon 1 PCR-amplified products generated from genomic DNA isolated from patient samples. Codon 13 mutation was determined by sequencing the K-*ras* exon 1 PCR-amplified products.

What is claimed is:

1. A method of reducing the viability of pancreatic cancer cells comprising contacting the cancer cells with an effective amount of an NSAID.

2. A method of increasing the susceptibility of mammalian pancreatic cancer cells to a chemotherapeutic agent comprising contacting the cells with an effective sensitizing amount of an NSAID.

3. The method of claim 1 or 2 wherein the NSAID is sulindac or an analog thereof that is a COX-2 inhibitor.

4. The method of claim 1 or 2 wherein the mammalian cancer cells are human cancer cells.

5. The method of claim 3 wherein the sulindac or the analog thereof is administered to a human cancer patient.

6. The method of claim 5 wherein the cancer patient is undergoing treatment with a chemotherapeutic agent.

7. The method of claim 6 wherein the chemotherapeutic agent is gemcitabine.

8. The method of claim 3 wherein the sulindac or the analog thereof is administered orally.

9. A method of evaluating the ability of sulindac or an analog thereof that is a COX-2 inhibitor to sensitize pancreatic cancer cells to a chemotherapeutic agent comprising:

(a) isolating a first portion of pancreatic cancer cells from a human pancreatic cancer patient;

(b) measuring their viability;

(c) administering sulindac or the analog thereof to said patient;

(d) isolating a second portion of pancreatic cancer cells from said patient;

(e) measuring the viability of the second portion of pancreatic cancer cells; and

(f) comparing the viability measured in step (e) with the viability measured in step (b); wherein reduced viability in step (e) indicates that the cells have been sensitized to said chemotherapeutic agent.

10. The method of claim 9 wherein steps (b) and (e) are carried out in the presence of the chemotherapeutic agent.

11. The method of claim 10 wherein the chemotherapeutic agent is gemcitabine and/or 5-FU.

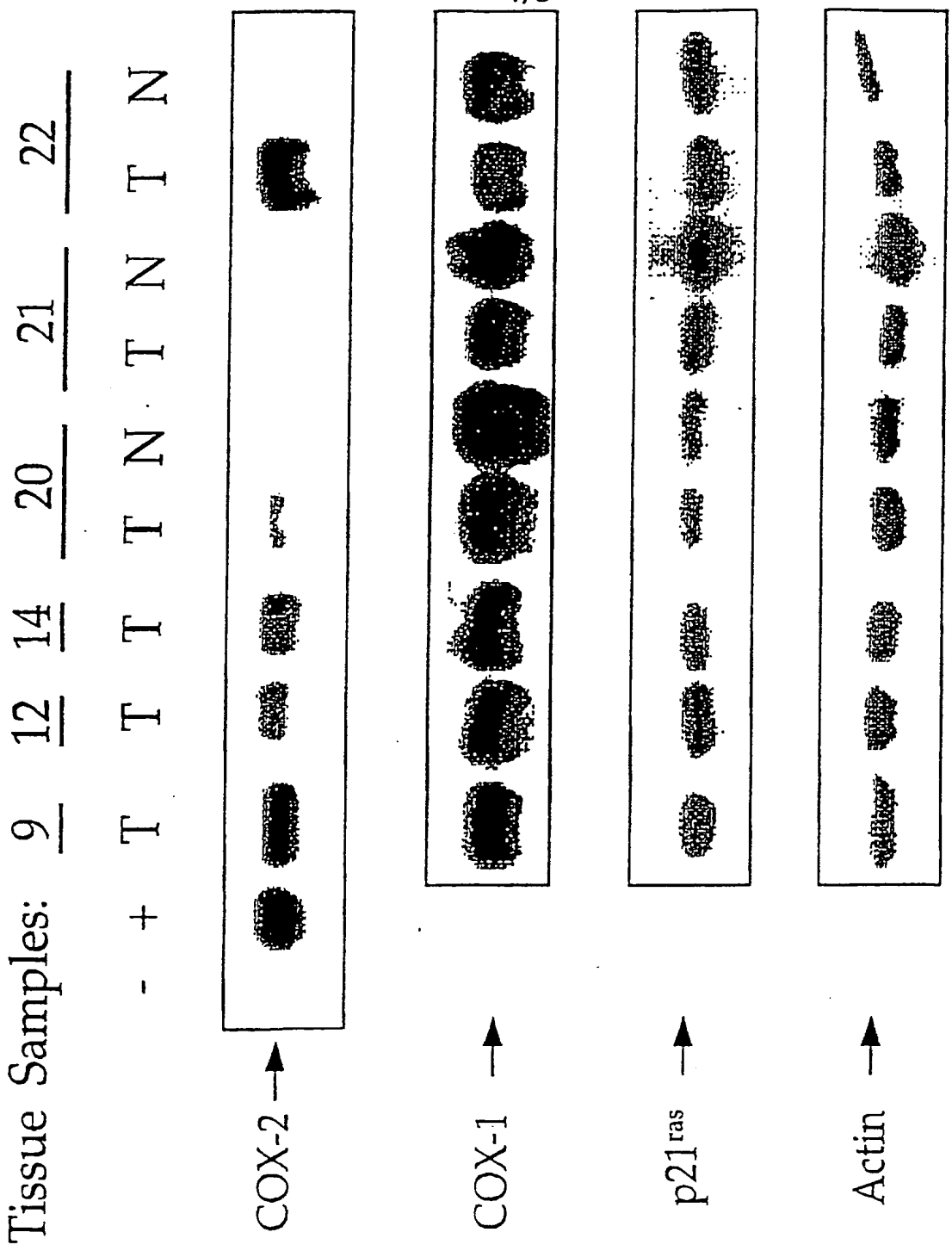


FIG. 1

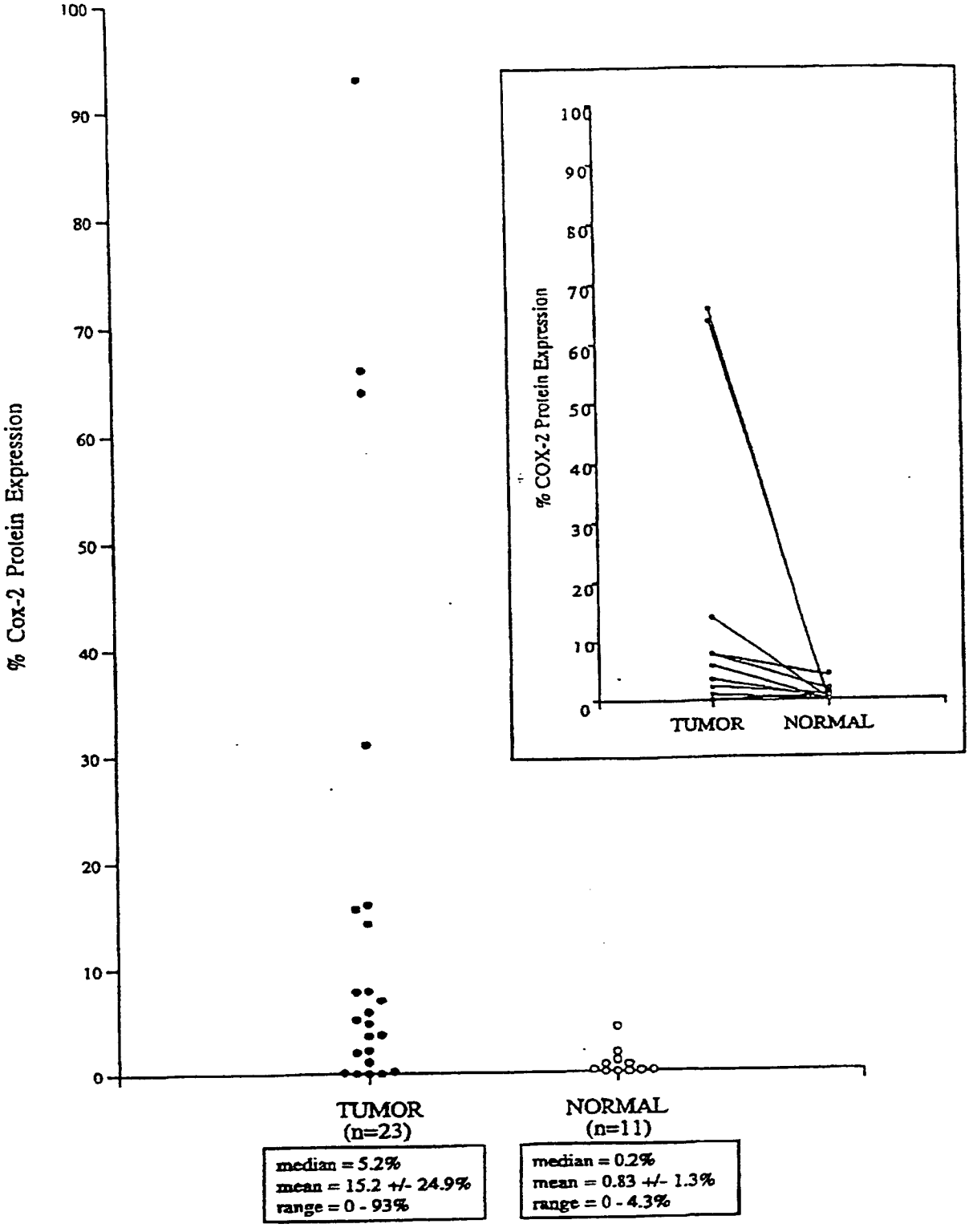

% Cox-2 Protein Expression
100
90
80
70
60
50
40
30
20
10
0
TUMOR
(n=23)
median = 5.2%
mean = 15.2 +/- 24.9%
range = 0 - 93%
NORMAL
(n=11)
median = 0.2%
mean = 0.83 +/- 1.3%
range = 0 - 4.3%
% COX-2 Protein Expression
TUMOR
NORMAL

FIG. 2

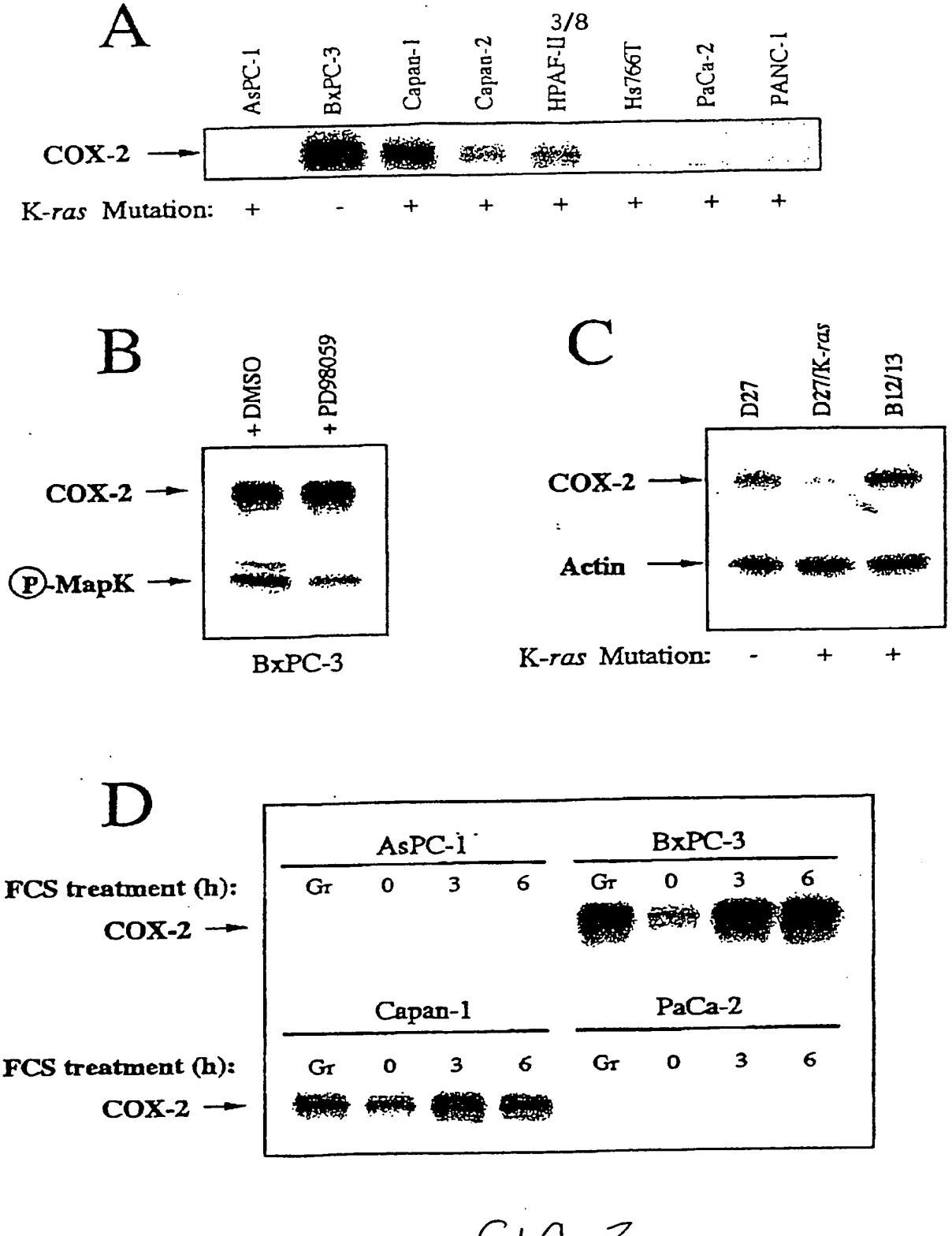
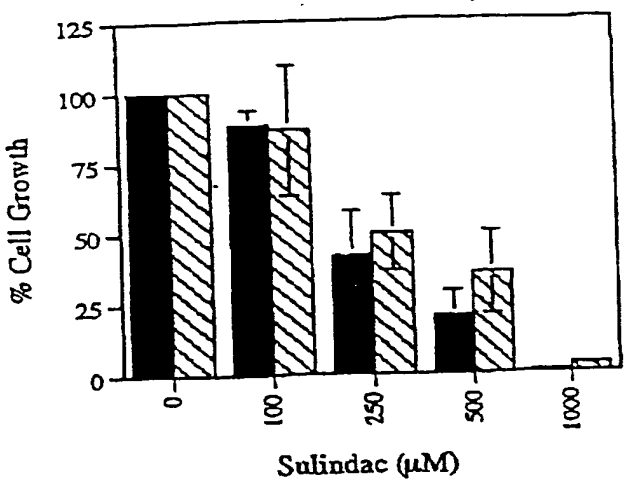
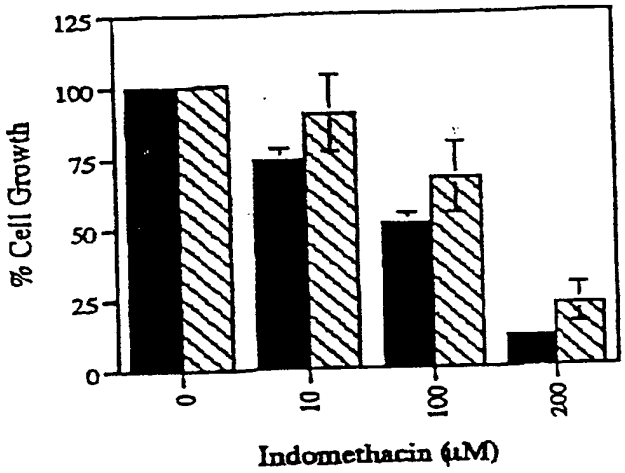

A
AsPC-1
BxPC-3
Capan-1
Capan-2
HPAF-II
Hs766T
PaCa-2
PANC-1
COX-2
K-ras Mutation: + - + + + + + +
B
+ DMSO
+ PD98059
COX-2
P-MapK
BxPC-3
C
D27
D27/K-ras
B12/13
COX-2
Actin
K-ras Mutation: - + +
D
AsPC-1
BxPC-3
FCS treatment (h):
Gr 0 3 6
COX-2
Capan-1
PaCa-2
FCS treatment (h):
Gr 0 3 6
COX-2

FIG. 3

A


125
100
75
50
25
0
% Cell Growth
0
100
250
500
1000
Sulindac (μM)

B


125
100
75
50
25
0
% Cell Growth
0
10
100
200
Indomethacin (μM)

C

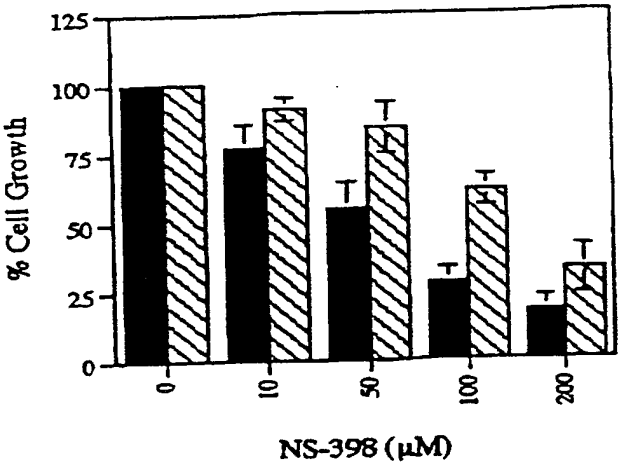

125
100
75
50
25
0
% Cell Growth
0
10
50
100
200
NS-398 (μM)

FIG. 4

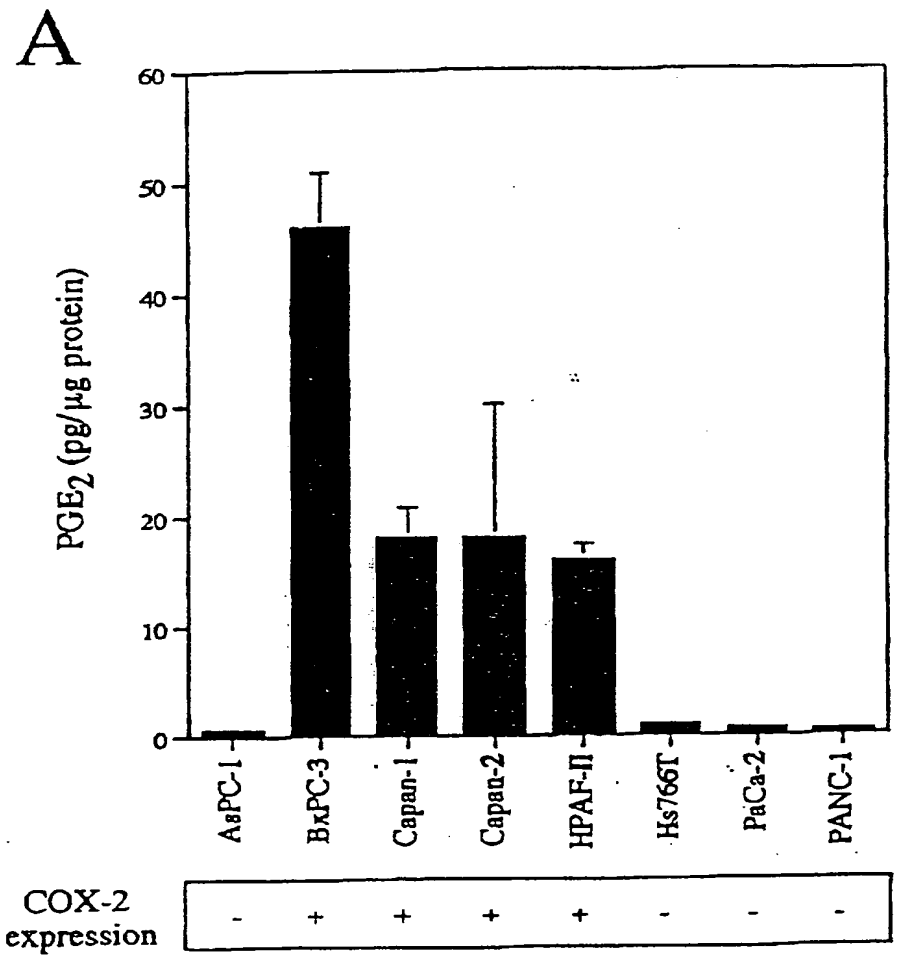
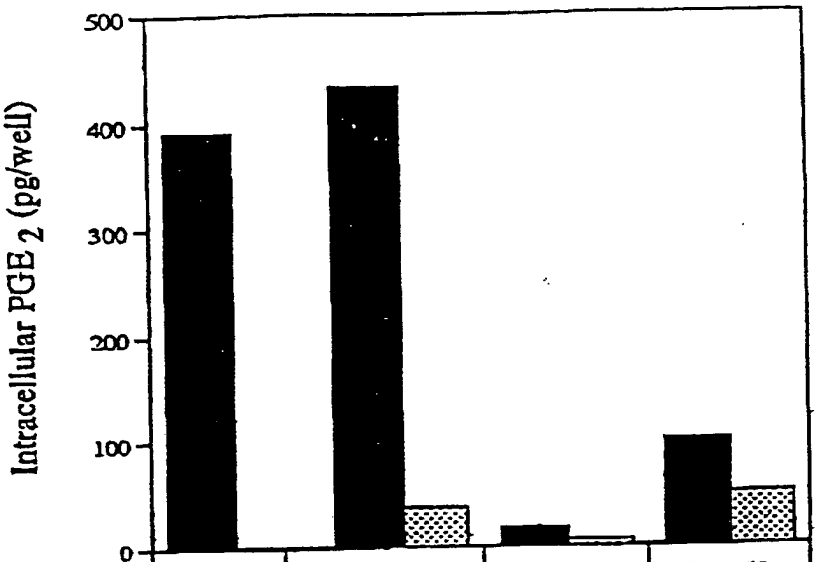


FIG. 5

B



Concentration:	-	100, 250 (μM)	10, 100 (μM)	10, 50 (μM)
Treatment:	DMSO	Sulindac	Indometh.	NS-398
% inhibition:	0	0, 90	95, 98	75, 86

FIG. 5 (cont.)

Effect of Sulindac + Gemcitabine on the growth of the pancreatic tumor cell line, BxPC-3 (day 3)

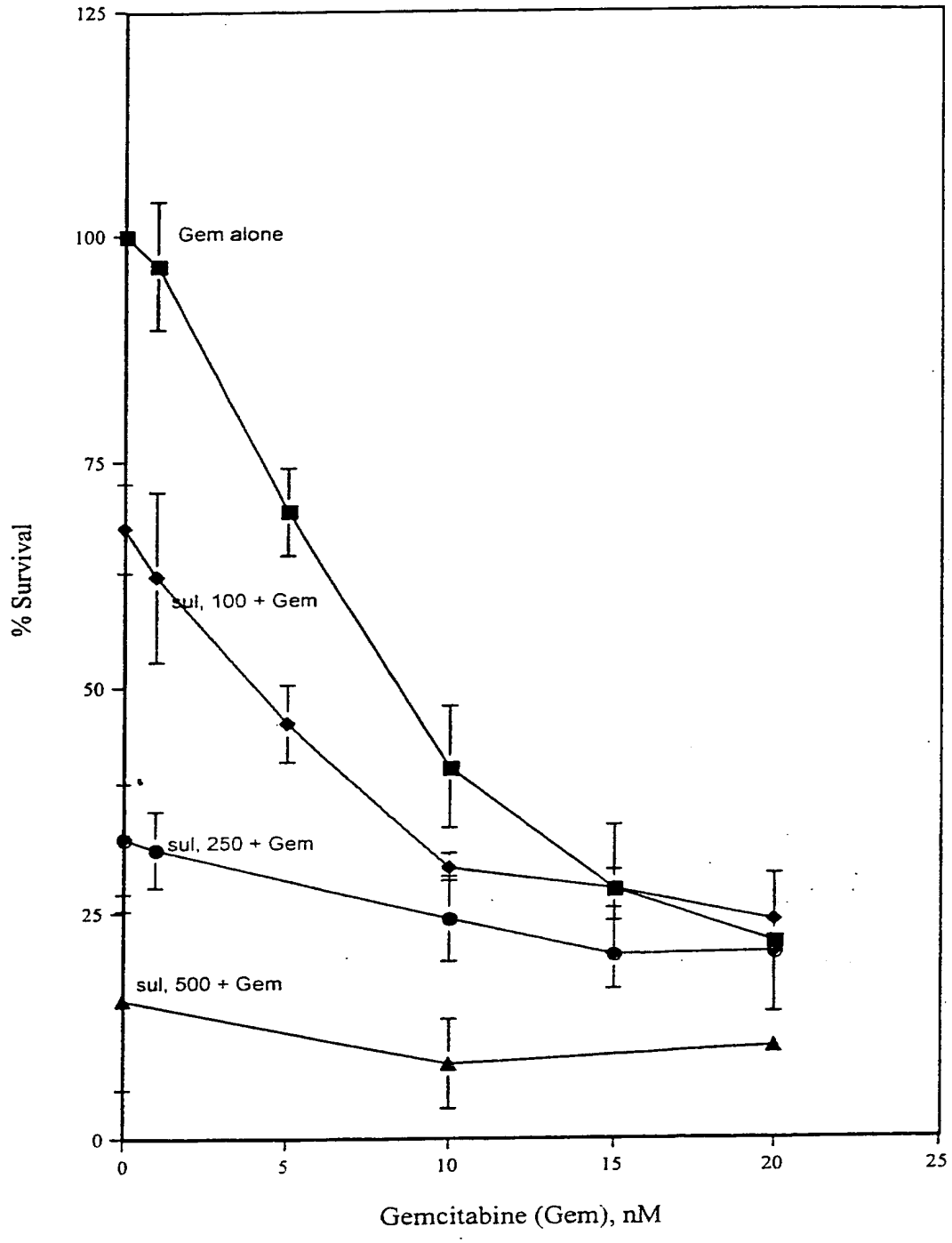


Fig. 6

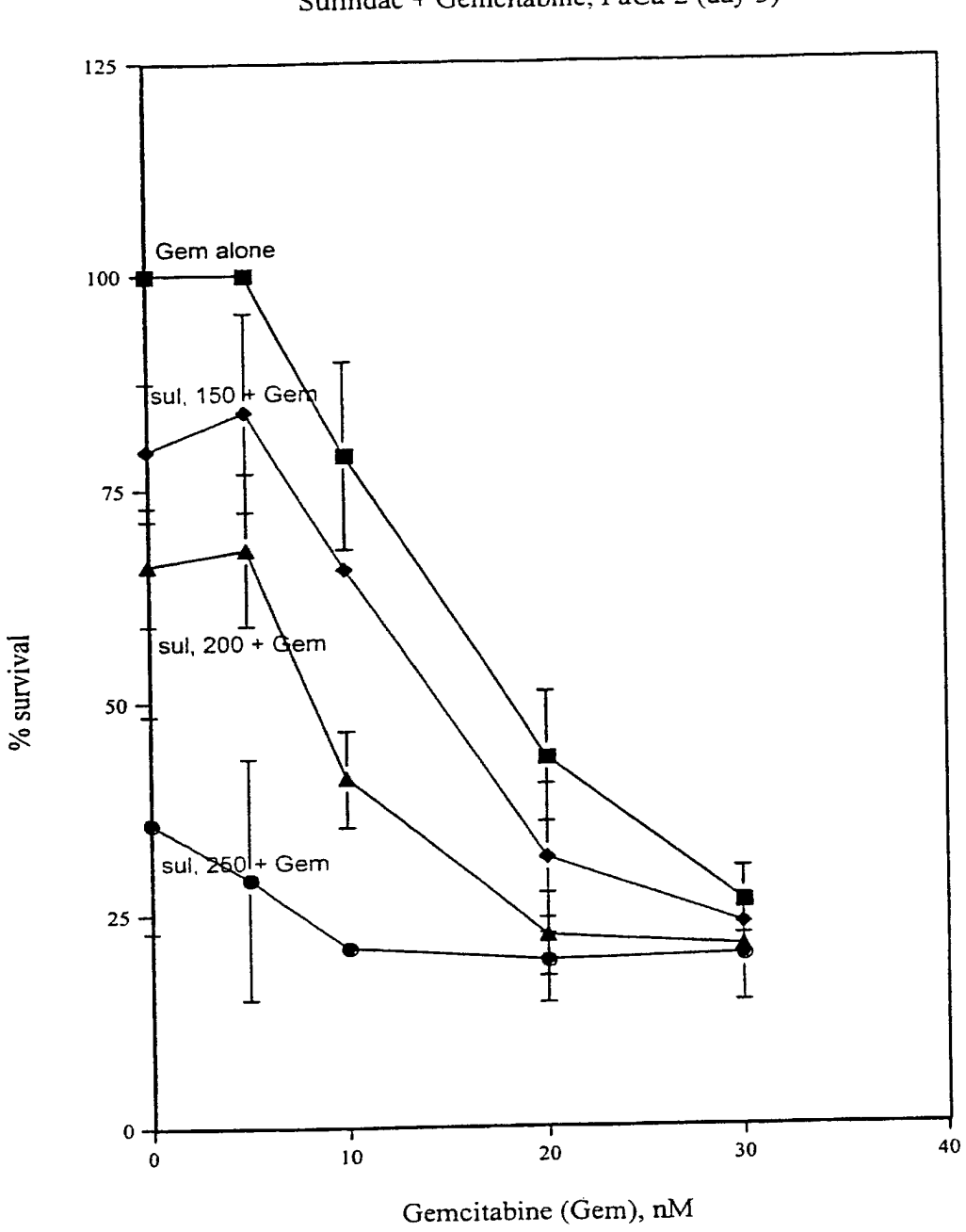

Sulindac + Gemcitabine, PaCa-2 (day 3)
125
100
75
50
25
0
% survival
Gem alone
sul, 150 + Gem
sul, 200 + Gem
sul, 250 + Gem
0
10
20
30
40
Gemcitabine (Gem), nM

Fig. 7

SEQUENCE LISTING

<110> Advanced Research and Technology Institute, Inc.
Marshall, Mark Steven
Sweeney, Christopher J.
Yip-Schneider, Michele T.
Crowell, Pamela L.

<120> Use of NSAIDs for the treatment of pancreatic cancer

<130> 740.018WO1

<150> US 60/165,543
<151> 1999-11-15

<160> 2

<170> FastSEQ for Windows Version 4.0

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<212> DNA
<213> Homo sapiens

<400> 1
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<212> DNA
<213> Homo sapiens

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ctctattgtt ggatcatatt 20

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/31410

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/19 A61K31/405 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, CHEM ABS Data, MEDLINE, EMBASE, BIOSIS, CANCERLIT

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	SWEENEY J. ET AL.: "INHIBITION OF CELL GROWTH IN PANCREATIC TUMOR CELLS BY ANTI-INFLAMMATORA DRUGS" PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH, vol. 41, March 2000 (2000-03), page 527 XP002164391 USA ABSTRACT #3358 abstract --- -/--	1-11

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 March 2001	18.04.01

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Economou, D

1

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	MARSHALL M.S. ET AL.: "SUPPRESSION OF PANCREATIC DUCTAL ADENOCARCINOMA GROWTH BY SULINDAC" PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH, vol. 41, March 2000 (2000-03), page 526 XP002164392 USA ABSTRACT #3349 abstract	1-11
P,X	T.YIP-SCHNEIDER M. ET AL.: "COX-2 EXPRESSION IN HAMAN PANCREATIC ADENOCARCINOMAS" CARCINOGENESIS, vol. 21, no. 2, February 2000 (2000-02), pages 139-146, XP000984815 the whole document	1-11
X	MOLINA M. ET AL.: "INCREASED COX-2 EXPRESSION IN HUMAN PANCREATIC CARCINOMAS AND CELL LINES: GROWTH INHIBITION NY NONSTEROIDAL ANTI-INFLAMMATORY DRUGS" CANCER RESEARCH, vol. 59, no. 17, September 1999 (1999-09), pages 4356-4362, XP000984712 the whole document	1-11
X	WO 99 49859 A (THE ARIZONA BOARD OF REGENTS) 7 October 1999 (1999-10-07) claims 1-21,6	1-6,8
X	WO 97 48391 A (ADVANCED RESEARCH AND TECHNOLOGY INSTITUTE) 24 December 1997 (1997-12-24) page 9, line 18 -page 11, line 7 claims 1-19	1-8
P,X	WO 00 38730 A (G.D.SEARLE & CO.) 6 July 2000 (2000-07-06) claims 1-99 example 6	1-8

INTERNATIONAL SEARCH REPORT

ormation on patent family members

International Application No
PCT/US 00/31410

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9949859 A	07-10-1999	AU 3366399 A	18-10-1999
		EP 1067919 A	17-01-2001
WO 9748391 A	24-12-1997	AU 3641597 A	07-01-1998
WO 0038730 A	06-07-2000	AU 2207000 A	31-07-2000
		AU 2209800 A	31-07-2000
		AU 2210400 A	31-07-2000
		AU 2380500 A	31-07-2000
		AU 2592600 A	31-07-2000
		AU 2593600 A	12-07-2000
		AU 2713400 A	31-07-2000
		AU 2713500 A	31-07-2000
		AU 2713600 A	31-07-2000
		WO 0038715 A	06-07-2000
		WO 0038716 A	06-07-2000
		WO 0038665 A	06-07-2000
		WO 0038717 A	06-07-2000
		WO 0038786 A	06-07-2000
		WO 0038718 A	06-07-2000
		WO 0038719 A	06-07-2000
		WO 0037107 A	29-06-2000